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(71) Applicant: NEW YORK UNIVERSITY [US/US]; 70 Washington Square South, New York, NY 10012 (US).

(72) Inventors: CERAMI, Carla ; 333 East 30th Street, New York, NY 10016 (US). FREVERT, Ute ; 545 First Avenue, New York, NY 10016 (US). SINNIS, Photini ; 3 Washington Square Village, New York, NY 10012 (US). NUSSENZWEIG, Victor ; 110 Bleeker Street, New York, NY 10012 (US).

(74) Agents: ROBINSON, Joseph, R. et al.; Darby & Darby, 805 Third Avenue, New York, NY 10022 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR INHIBITING HEPATOCYTE INVASION BY MALARIAL SPOROZOITES

(57) Abstract

This invention is directed to compositions and methods for inhibiting hepatocyte invasion by malarial sporozoites. More specifically, the invention is directed (a) to ligands for the hepatocyte plasma membrane receptor for the circumsporozoite protein and peptides based on a portion of the circumsporozoite protein that constitutes an essential part of the specific ligand for this receptor; and (b) to methods using such peptides to inhibit circumsporozoite invasion of liver cells.

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COMPOSITIONS AND METHODS FOR INHIBITING HEPATOCYTE INVASION BY MALARIAL SPOROZOITES

The United States Government has rights to this invention by virtue of the following grants: Grant No. 5T32GM07308 from the National Institutes of Health; NIH5T 32CA9161-16; and Grant No. DPE-0453-A-00-5012-00 from the Agency for International Development.

FIELD OF THE INVENTION

This invention is directed to compositions and methods for inhibiting hepatocyte invasion by malarial sporozoites. More specifically, the invention is directed (a) to ligands for the hepatocyte plasma membrane receptor for the circumsporozoite protein and peptides based on a portion of the circumsporozoite protein that constitutes an essential part of the specific ligand for this receptor; and (b) to methods using such peptides to inhibit circumsporozoite invasion of liver cells.

BACKGROUND OF THE INVENTION

Malaria is transmitted by the bite of the Anopheles mosquito. Minutes after infection, sporozoites (the mosquito-hosted stage of the malarial parasite) enter hepatocytes of the susceptible mammal where they multiply by schizogony and develop into exoerythrocytic forms ("EEF"). Except in highly endemic areas, the number of parasites inoculated by a single mosquito is small, probably below 100 but malarial infection has high efficiency. This coupled with the uniqueness of the target (the victim's liver cells) suggests that hepatocyte invasion is receptor-mediated. However, neither the structure of the receptors nor that of the ligands had been elucidated.

The circumsporozoite protein, a malarial stage- and species-specific protein that uniformly covers the surface

membrane of sporozoites isolated from mosquito salivary glands and constitutes one of the main proteins expressed by mature infective sporozoites would be a candidate ligand for a hepatic cell receptor if such a receptor existed.

The circumsporozoite (CS) protein has been extensively investigated (reviewed in Nussenzweig and Nussenzweig, Adv. Immunol. 45:283-334, 1989). The sequences of CS proteins from several malarial species have been elucidated and their main structural and antigenic properties which show substantial interspecies similarities have been described in Doolan et al., Infect. Immunol. 60:675-682, 1992; Lockyer et al., Mol. Biochem. Parasitol. 37:275 (1989); De La Cruz et al., J. Biol. Chem. 262:11925-11940, 1987; McCutchan et al., Science 230:1381-1383, 1985; Lal et al., Mol. Biochem. Parasitol. 30:291-294, 1988; Godson, et al., Nature 305:29-33, 1983; Galinsky et al., Cell 48:311-319 (1987); Lal et al., J. Biol. Chem. 263:5495-5498, 1988; Eichinger et al., Mol. Cell. Biol. 6:3965-3972, 1986; Lal et al., J. Biol. Chem. 262:2937-2940, 1987; and Hedstrom et al., WHO Bulletin OMS (Suppl.) 68:152-157, 1990. See also U.S.P.N. 4,915,942; U.S.S.N. 07/370,241 filed June 22, 1989, now allowed; U.S.S.N. 07/099,652, filed September 21, 1987, now abandoned; and U.S.S.N. 07/864,172, filed April 3, 1992.

All CS proteins contain (i) a species-specific immunodominant repeat domain encompassing about one-half of their molecule; (ii) two pairs of cysteines in the C-terminal region, and (iii) two relatively short stretches of conserved amino acid sequences flanking the repeat domain.

The N-terminal proximal conserved sequence (Region I) is the smaller of the two conserved regions and has been described in Dame, J.B. et al., Science 225:593-599, 1984. One group of investigators reported that peptides corresponding to Region I bind to hepatocytes and that antibodies against this region inhibit invasion (Aley, S.B. et al., J. Exp. Med. 164:1915-1921, 1986) but to the knowledge of the present

inventors, there has been no follow up or independent confirmation of these studies.

The C-terminal proximal conserved sequence (Region II) surrounds the first pair of cysteines on the C-terminal side of the repeat domain. Region II was initially described by Dame et al., supra, but has now been redefined by the present inventors and as redefined will hereafter be referred to as Region II+. Region II+ is highly homologous to a cell-adhesion domain of thrombospondin (Prater et al., J. Cell. Biol. 112:1031-1040. 1991; Tuszynski, G.P. et al., Exp. Cell. Res. 182:473-481, 1989) as well as to regions of several other proteins such as properdin, von Willebrand factor and antistasin, the latter being a leech anti-coagulant (Clarke, L.E. et al., Mol. Biochem. Parasitol. 41:269-280, 1990; Hedstrom, R.C. et al., WHO Bulletin OMS (Suppl.) 68:152-157, 1990; Robson, K.J.H. et al., Nature 335:79-82, 1988; Goundis, D. et al., Nature 335:82-85, 1988). Region II as defined by Dame et al. did not prove to be immunogenic and was discarded as a candidate for a malaria vaccine. No function was attributed to it. CS regions adjacent to Region II, however, were shown to be immunogenic (See, e.g., U.S.P. 4,915,942). Region II+ was redefined based on extensive homology, considering not only P. knowlesi and P. falciparum (as Dame et al. had done) but also considering many other malarial species. See, Table 1 below.

Although the CS proteins have been extensively investigated and a large amount of information has been accumulated on their structure, immunological properties and evolution, their function remains unknown. The participation of the CS protein in hepatocyte invasion has been suggested by the observation that Fab fragments of monoclonal antibodies against the repeats inhibit sporozoite infectivity in vitro and in vivo. However, the ligand (if any) recognized by hepatocyte receptors did not seem to be in the repeats, in view of the fact that, for example, sporozoites of different species (the CS proteins of which have different repeat units) infect the liver of the same

host. Moreover, immunization of hosts (especially human hosts) with synthetic repeat peptides abolished infectivity of sporozoites when relatively high levels of antibodies were elicited. Nevertheless, even small amounts of antibodies eliminated a large portion, but not all infected sporozoites. Repeat peptides can attenuate the severity of subsequent malarial infection, and thus repeat peptides have utility.

Attempts to isolate or identify the hepatocyte receptor or its corresponding ligand in sporozoites have not borne any results to date.

Malaria currently afflicts 270 million new human victims every year and accounts for nearly two million yearly deaths. In many parts of the world where malaria is endemic the parasites are resistant to all known chemotherapeutic drugs and there is evidence that resistance is spreading. Many investigators are currently involved in the development of vaccines against the sporozoite and the merozoite stage of malaria. Progress is being made but it is slow. As a result, malaria continues to threaten large numbers of the world's population. Malaria is most lethal against travellers who (unlike natives of endemic areas) have no partial immunity to the disease. Partial immunity is acquired from continuous exposure to infected mosquitoes.

OBJECTS OF THE INVENTION

Objects of the invention include the discovery of novel agents and methods that are useful in inhibiting circumsporozoite protein binding to and sporozoite invasion of hepatocytes and in designing drugs and agents useful for the same purposes. especially for chemo-prophylaxis as well as immuno-prophylaxis against malaria.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a peptide inhibitor for the binding of a circumsporozoite polypeptide to receptors of hepatocytes from

malaria-susceptible mammals. The inhibitor has an amino acid sequence selected from the group consisting of:

(i) Region II+ of a circumsporozoite protein, the Region II+ containing the subsequence CSVTCG;

(ii) fragments of the Region II+ containing at least a portion of the adhesion ligand for the receptors, the portion comprising at least one cysteine of the Region II+;

(iii) peptide constructs comprising (a) (i) or (ii) and (b) at least one other fragment of the amino acid sequence of the circumsporozoite protein, the constructs having no substantial ability to elicit the formation of antibodies recognizing the immunodominant epitope of the circumsporozoite protein.

In the other embodiment, a peptide inhibitor for the binding of a circumsporozoite polypeptide to basolateral plasma membrane of hepatocytes from malaria-susceptible mammals is provided. The inhibitor has an amino acid sequence selected from the group consisting of:

(i) Region II+ of a circumsporozoite protein, the Region II+ containing the subsequence CSVTCG;

(ii) fragments of the Region II+ containing at least a portion of the adhesion ligand for the receptors, the portion comprising at least one cysteine of Region II+;

(iii) peptide constructs comprising (a) (i) or (ii) and (b) at least one other fragment of the amino acid sequence of the circumsporozoite protein, the constructs having no substantial ability to elicit the formation of antibodies recognizing the immunodominant epitope of the circumsporozoite protein.

Also contemplated is a method of inhibiting the binding of a circumsporozoite polypeptide to hepatocytes susceptible to sporozoite invasion comprising:

supplying to the environment of the hepatocytes an amount effective to inhibit the binding of the peptide inhibi-

tor(s) above, no later than exposure of the hepatocytes to the circumsporozoite protein.

A peptide consisting essentially of Region II+ of the circumsporozoite protein is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of various CS recombinant polypeptides.

Figure 2: Shows photomicrographs illustrating the binding of CS polypeptides to sinusoids in liver sections or to human hepatocyte cell line HepG2. Binding is revealed with anti-repeat MAb 2A10 followed by a conjugate of rat anti-mouse IgG conjugated to fluorescein isothiocyanate.

Panel 2A: Binding of 12.5 μg/ml CS271VC (control);
Panel 2B: Binding of 12.5 μg/ml falciparum-1;
Panel 2C: Inhibition of binding of CS271VC by 250 μg/ml PfRII+;
Panel 2D: No inhibition of binding of CS271VC by 500 μg/ml Pf3;
Panel 2E: Binding of CSFZ to HepG2.

Figure 3: Panel A shows photomicrographs of the binding of CS polypeptides to human hepatocyte microvilli within the Space of Disse (arrows or lateral membranes of adjacent hepatocytes (arrowheads)), but no binding in bile canaliculi (BC) or endothelia cells (EC).

H: hepatocyte
N: nucleus of hepatocyte
S: sinusoid
E: erythrocyte

Figure 3: Panel B shows a higher magnification of the Space of Disse of 3A showing aggregates of CS polypeptide (CS27IVC) binding to hepatocyte microvilli. Binding is revealed as in Fig. 2 but with gold instead of fluorescein.

Figure 4: Shows photomicrographs of localized CS-binding sites in human and rat liver cells. Letters not defined above have the following significance: D=Disse space; L=lysosomes; K=Kupffer cell; *=contaminating cell organelle.

Panel A: binding of gold labelled CS27IVC human liver;

Panel B: binding of CS27IVC to rat liver -- aggregated gold particles are shown under lysosomes of K but not on K surface;

Panel C: binding of CS27IVC to rat liver cell membrane fractions;

Panel D: non-binding of CS27IVC to rat liver mitochondrium and rough endoplasmic reticulum.

Figure 5: Top panel shows the FPLC elution profile of CS27IVC following passage through a Superose column. Ordinate: $ODx10^{-2}$; Abscissa: fraction number.

Bottom panel shows aggregates of CS in fraction 9 and monomers of CS in fractions 12-14. Molecular weight markers shown on top.

Figure 6: A graph showing binding of CS to HepG2 cells. Fluorescence indicates amounts of bound protein.

Figure 7: A graph of percent inhibition by RII+ peptides of the binding of CSFZ (Cys) to HepG2 cells as a function of RII+ concentration.

DETAILED DESCRIPTION OF THE INVENTION

All literature, patents, applications and other published documents cited herein are incorporated by reference in their entirety.

Materials

Recombinant P.vivax and P.falciparum proteins and polypeptide fragments thereof can be made, e.g. in accordance with now well-known recombinant techniques, see, e.g., Barr, P.J. et al., J. Exp. Med. 165:1160-1171, 1987 and U.S.P. Nos. 4,997,647 and 4,880,734 and European Patent Publication No. 460716. Their sequences as well as other recombinant methods for making them have been published in Dame, J.B. et al., Science 225:593-599, 1984; and McCutchan, T.F. et al., Science 230:1381-1383, 1985. Other CS sequences have been published in Doolan, D.L. et al., Infect. Immunol. 60:675-682, 1992, Lockyer, M.J. et

al., Mol. Biochem. Parasitol. 37:275, 1989; De La Cruz, V.F. et al., J. Biol. Chem. 262:11935-11940, 1987; Galinsky, M.R. et al., Cell 48:311-319, 1987; Eichinger, D.J. et al., Mol. Cell. Biol. 6:3965-3972, 1986, Lal, A.A. et al., Mol. Biochem. Parasitol. 30:291-294, 1988; and Hedstrom, R.C. et al., WHO Bulletin OMS (Suppl.) 68:152-157, 1990.

The following peptide fragments of CS proteins were used in experiments:

Vivax-2: Includes the entire N-terminal moiety of P.vivax CS protein, the repeats Region II+ (as redefined by the present inventors) and 11 amino acid residues downstream from the end of the repeats through a leucine residue. Vivax-2 has no free sulfhydryl groups as determined by Ellman reaction.

Falciparum-2: The corresponding fragment of P.falciparum CS protein,also terminating at the leucine residue (P.falciparum CS residues 43-391). Falciparum-2 also has no free sulfhydryl groups as determined by Ellman reaction.

Vivax-1: Same as Vivax-2 minus most of Region II+, terminating with the proline residue immediately preceding the first cysteine of Region II+.

Falciparum-1: Same as Falciparum-2 minus most of Region II+, terminating with the proline residue of preceding the first cysteine of Region II+ (P.falciparum residues 43-348).

See Figure 1 for more details on the foregoing polypeptides.

Region II+: Region II in P.falciparum has been redefined by the present inventors as:

E-W-S-P-C-S-V-T-C-G-N-G-I-Q-V-R-I-K

The corresponding Regions II+ for other malarial species and the extensive homology among them are shown in Table 1 below.

TABLE 1

SPECIES	SEQUENCE
P. falciparum	PSDKHIEQYLKKIKNSISTQ KK NR I L E QT Q T R E W S P C S V T C G N G I Q V R I K P G S A D S Q A NKDPDELDYENDIE G
P.vivax	E W T P C S V T C G V G V - - R V R
P.malariae	E W S P C S V T C G S G I R A R R K
P.knowlesi	E W S P C S V T C G K G V R M R R K
P.cynomolgi	E W S P C S V T C G K G V R M R R K
P.brasilianum	E W S P C S V T C G S G I R A R R K
P.berghei	E W S Q C N V T C G S G I R V R K R
P.yoelii	E W S Q C S V T C G S G V R V R K R
P.yoelii (SSP-2)	E W S E C S T T C D E G R K I R R R

In addition. the following peptides and polypeptides were synthesized:

CS27IVC: Consists from N- to C-terminal of residues 27-123 plus $(NANPNVDP)_3$ plus $(NANP)_{21}$ plus residues 300-411 of the P.falciparum CS protein. See, Takacs et al., J. Immunol. Meth. 143:231-240, 1991; Hochuli et al., J. Chromat. 411:177-184, 1987.

CSFZ(Cys): Consists of residues 27-123 plus NANP (a single instance) plus residues 300-411 of P.falciparum CS protein.

In addition, the following P.falciparum derived peptides were constructed as described in Houghten, R.A., Proc. Nat'l. Acad. Sci. USA 82:5131-5135, 1985.

Pf1	PCSVTCGNGIQVRIKPGSAN
Pf1A	PASVTAGNGIQVRIKPGSAN
Pf1B	PXSVTXGNGIQVRIKPGSAN
Pf2	CGNGIQVRIKPGSANKPKDE
Pf2A	AGNGIQVRIKPGSANKPKDE
Pf3	PGSANKPKDELDYANDIEKK
PbRII+	CNVTCGSGIRVRKRKGSNKKAEDL
Pf70	KPKHKKLKQPADGNPDPNAN
Pf4	PGSANKPKDELDYANIEKK

Pf1: contains most of P.falciparum Region II+.

Pf1A: Identical to Pf1 except alanine residues are substituted for the two cysteines.

Pf1B: Identical to Pf1 except the sulfhydryl groups on both cysteines have been blocked with acetamide groups.

Pf2: Contains most of Region II+.

Pf2A: Identical to Pf2 except the first cysteine has been replaced by an alanine residue.

Pf25C: A scrambled version of Pf2.

Pf3: Contains only a C-proximal moiety of Region II+.

PbRII+: Contains Region II+ from P.berghei

Pf70: Contains most of P.falciparum Region I.

Pf4: Contains only a C-proximal moiety of Region II+ except for a single amino acid deletion.

Antibodies

Monoclonal antibody 2A10 (prepared according to Nardin, E.H. et al., J. Exp. Med. 156:20-30, 1982) is directed against an epitope contained in the repeat - $(NANP)_n$ - region of P.falciparum CS protein and recognizes the amino acid sequence PDPNANPN found 5' of region II in the repeat-less recombinant polypeptide CSFZ (Cys) (Burkot, T.R. et al., Parasite Immunol. 13:161-170, 1991).

Monoclonal antibody 2E6 reacts with the liver stage of P.berghei. Such antibodies can be made by methods known to those skilled in the art.

Polyclonal antisera against P.berghei CS Region II were raised by immunizing rabbits with peptide PbRII coupled to key hole limpet hemocyanin with glutaraldehyde. A rabbit was immunized once with 500 μg of the peptide-adjuvant conjugate in complete Freund's adjuvant and then boosted monthly (four times) with the same amount of conjugate in incomplete Freund's adjuvant. The antisera recognize the peptide PbRII+ dried onto plastic wells, and this binding is inhibited by soluble PbRII+. The difficulty of making such antisera testifies to the non-immunogenic character of Region II+.

The invention is described in more detail below by reference to specific examples, which are only illustrative and not limiting in nature.

Example 1: Staining of Frozen Tissue Sections

The procedure was carried out essentially as described by Dukor P. et al., Proc. Natl. Acad. Sci. USA 67:991-997, 1970 and Imai Y. et al., J. Cell. Biol. 111:1225-1232, 1990. Rats were euthanized with CO_2. Small pieces of the liver, spleen, heart, brain and lung were removed, snap frozen in liquid nitrogen, embedded in Tissue Tek O.C.T. (Miles, Inc., Naperville, Illinois), and cut into 5 μm sections. Sections were dried for

30 min, fixed for 10 min. in 4% paraformaldehyde, rinsed three times with phosphate-buffered saline (PBS), and either used immediately or stored at 4°C in 1% BSA, 0.5% Tween, PBS (BSA/TPBS). After blocking with 100 mM glycine (pH 7.2) and with BSA/TPBS, the sections were sequentially incubated at 37°C with recombinant proteins for 1 hr, 10 μg/ml MAb 2A10 for 45 min., and rat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (Boehringer Mannheim, Indianapolis, Indiana) for 45 min. The slides were counterstained for 10 min. with 0.3% Evans blue, rinsed extensively in PBS, and finally observed under a fluorescence microscope. For peptide inhibition experiments, the sections were first incubated with the peptide at various concentrations in BSA/TPBS for 1 hour at 37°C, washed six times with BSA/TPBS, and then stained with 2.5 μg/ml CS recombinant protein as described above. In a few experiments, the tissues were fixed for 10 min. with methanol, acetone, or 4% paraformaldehyde containing 0.5% glutaraldehyde.

Example 2: Isolation of Hepatocyte Membranes

Fractionation of rat liver cells was performed as described by Hubbard A.L. et al., J. Cell. Biol. 96:217-229, 1983. In brief, perfused rat livers were homogenized and subjected to sucrose gradient centrifugation. The membrane preparation and the pellet, consisting mostly of mitochondria and rough endoplasmic reticulum, from the final centrifugation step were processed for ultrastructural examination.

Example 3: Electron Immunomicroscopy

Rat or mouse liver tissue or hepatocyte subcellular fractions from Example 2 were fixed in PBS containing 1% glutaraldehyde (grade 1, Sigma, St. Louis, Missouri) and 4% paraformaldehyde (Kodak, Rochester, New York), dehydrated in ethanol, and embedded in LR White (Polysciences, Warrington, Washington). Frevert et al., Infect. and Immun. 60:2349-2360, June 1992. Normal human liver was embedded in Lowicryl K4M (Ted Pella, Redding, CA). Ultrathin sections were labelled by incubating them sequentially with 10-50 μg/ml CS271V, CSFZ (Cys), Falc-2, or Falc-1 for 30 min.; 15 μg/ml MAb 2A10 for 30 min.;

protein A-gold 15 nm (PAG15, 1:30; Amersham, Arlington, Illinois) or goat anti-mouse IgG gold 10 nm (GAM10, 1:30; Amersham) for 30 min. Control specimens were incubated in the absence of CS and only with the gold conjugates. Photographs were taken with a Philips EM 301 electron microscope.

Example 4: HepG2 Cell Binding Assay

For indirect immunofluorescence, HepG2 cells (ATCC number HB8065, Rockville, Maryland; Knowles, B.P., et al., Science 209:497-499, 1980) were grown on slides (Cel-Line Associates, Inc., Newfield, New Jersey) overnight in minimum essential medium with 10% fetal calf serum (FCS-MEM; GIBCO, Grand Island, New York), 1 mM L-glutamine (GIBCO), 3 mg/ml glucose (Sigma), 1 x nonessential amino acids (GIBCO), 50 μg/ml penicillin, and 100 μg/ml streptomycin (GIBCO). For the enzyme-linked immunosorbent assay, 10^5 HepG2 cells were deposited in 96-well Falcon tissue culture plates (Becton Dickinson, Oxnard, California) and grown for 24 hr. in FCS-MEM. The cells were fixed with 4% paraformaldehyde, washed three times with PBS, and stored at 4°C in BSA/TPBS until use. Before the experiments, plates were blocked for 2 hr. at 37°C with 1% gelatin, 0.05% Tween in PBS (pH 7.4) (gelatin/TPBS). The cells were sequentially incubated at 37°C with 50 μl of recombinant protein diluted in gelatin/TPBS for 1 hr. MAb 2A10 at a concentration of 10 μg/ml for 30 min. and goat anti-mouse immunoglobulin conjugated to alkaline phosphatase (Boehringer Mannheim) for 30 min. Bound enzyme was revealed by the addition of the fluorescent substrate, 1 mM 4-methylumbelliferyl phosphate in 100 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$ (pH 9.5). After 15 min. fluorescence was read in a Fluoroskan II plate reader (Flow Lab Inc., McLean, Virginia) with excitation filter 350 nm and emission filter 460 nm. In the peptide inhibition experiments, wells were first incubated for 1 hr. at 37°C with peptides diluted in gelatin/TPBS, washed three times with gelatin/TPBS, and then incubated with recombinant CSFZ (Cys) at a concentration of 2.5 μg/ml for 1 hr. at 37°C. The bound CSFZ (Cys) was revealed as described below.

Example 5: FPLC Analysis of Recombinant CS Polypeptide

To separate monomers from multimers of recombinant CS, the preparations were subjected to molecular sieving chromatography on an FPLC apparatus (Pharmacia, Piscataway, New Jersey). One hundred micrograms of CS271VC in a phosphate buffer containing 150 mM NaCl (pH 7.2) was injected into a Superose 12 sizing column (Pharmacia). The protein was eluted in the same buffer using a flow rate of 0.2 ml/min. A high molecular weight fraction (69kD) co-eluted with thyroglobulin. The monomeric form of the protein eluted between amylase (molecular weight=200kD) and BSA (molecular weight=66kD).

Example 6: Western Blotting of Recombinant CS Polypeptides

Western blotting was conducted as described by Towbin, H.T. et al., Proc. Natl. Acad. Sci. USA 76:4350-4354, 1979. Aliquots of the fractions obtained from the FPLC analysis were run on a 7.5% SDS-polyacrylamide gel under nonreducing conditions and electrophoretically transferred to Immobilon membrane (Millipore Corporation, Bedford, Massachusetts). The nylon membrane was blocked for 30 min. with BSA/TPBS, incubated 1 hr. with 15 μg/ml MAb 2A10, washed three times with 0.5% Tween/OBS, and incubated with goat anti-mouse antibody coupled to alkaline phosphatase (Sigma). The bound enzyme was developed with bromochlorophenol blue and nitrotetrazolium blue. The results are shown in Figure 6B which illustrate that fraction 9 contained polymeric forms of the CS portein and fractions 12, 13 and 14 contained mainly monomers.

Example 7: Preparation of Falciparum-1 Aggregates

The methods described by Lambert, J.M. et al., Biochemistry 17:5406-5416, 1978 were followed. Falciparum-1 at a concentration of 1 mg/ml was incubated with 250 mM Traut's reagent (Pierce Chemical Co., Rockford, Illinois) in 50 mM triethylamine-hydrochloric acid (TEA-HCl) (pH 8.0), 1 mM $Mg(Ac)_2$, and 50 mM KCl for 20 min. on ice. Traut's reagent (0.5 M stock) was prepared immediately before use in a solution containing equal volumes of 1 M TEA-HCl and 1 M TEA-free base. The

sulfhydryl-containing falciparum-1 was oxidized in ambient air at 4°C overnight. The presence of aggregated disulfide-linked falciparum-1 was confirmed both by chromatography in an FPLC sizing column and by Western blotting as described above.

Example 8: Sporozoite Infection Assay

Assays were conducted according to the methods described by Hollingdale, M.R. et al.. Science 213:1021-1022, 1981. and Sinden, R.E., WHO Bulletin MOS (Suppl.) 68:115-125, 1990. HepG2 cells were plated in FCS-MEM at a density of 0.5 x 10^6 cells/ml in 8-chamber slides (4808 Lab-tek, Naperville, Illinois) 24 hr. before each experiment. For the peptide inhibition experiments, sporozoites were resuspended in FSC-MEM alone or FCS-MEM containing 500-125 μg/ml peptide. For antibody inhibition experiments, sporozoites were preincubated in FCS-MEM alone, or FCS-MEM containing anti-region II IgG, or with preimmune sera IgG for 30 min. at 4°C. Sporozoites (5 x 10^4), in a volume of 100 μl, were added to each well. The medium was replenished after 2 hr. and changed after 18 and 28 hr. Each point was performed in quadruplicate. In experiments designed to evaluate the toxicity of the peptides and antisera, sporozoites were first incubated with the HepG2 cells for 2 hr in FCS-MEM. FCS-MEM containing the peptide at a concentration of 250 μg/ml (or the IgGs at a concentration of 700 μg/ml) was then added to the cultures, and incubation proceeded for two additional hours. As above, FCS-MEM was changed after 18 and 28 hr. All cultures were fixed with cold methanol containing 0.3% H_2O_2 after 48 hr. Wells were blocked with BSA/TPBS, incubated for 45 min. with 10 μg/ml MAb 2E6 directed against the exoerythrocytic forms of the parasite, washed three times with BSA/PBS, incubated for 45 min. with goat anti-mouse immunoglobulin conjugated to horse-radish peroxidase (Accurate Chemical and Scientific Corp., Westbury, New York), and washed three times with BSA/PBS. Bound enzyme was revealed with 1 mg/ml 3,3'-diaminobenzidine in 0.05M Tris (pH 7.6), 0.01% $H_2)_2$. The number of exoerthyrocytic forms in 20

fields was counted in a double-blind fashion under a 20 x light microscope objective.

Example 9: Binding of CS in the Liver

Frozen sections of various rat organs were incubated with recombinant CS27IVC polypeptide. Tissue-bound CS was revealed with MAb2A10 and fluorescence microscopy. Strong staining was observed in liver sections with concentrations of CS27IVC within the range 50-5 μg/ml. The results are shown in Fig. 2A. The staining closely followed the sinusoidal spaces of the hepatic lobules, indicating CS binding to the hepatic membrane. Other areas of the liver sections and sections of other organs (spleen, lung, heart or brain) were not stained. Control sections (incubated with CS27IVC in the absence of antibody or vice versa) were not stained either.

The same pattern was observed using air-dried (instead of frozen) sections or in sections fixed with various fixtures (4% p-formaldehyde alone or in combination with 0.5% glutaraldehyde, acetone or methanol).

To define the region of the CS that mediates the perisinusoidal staining various other recombinant constructs were also used in the frozen section assay. The results were as follows:

CSFZ (which contains Region II and Region I but only one repeat copy) bound with the same pattern as CS27IVC;

Falciparum-2 (which contains Region I and II as well as the repeats) bound to the liver sections at 25 μg/ml;

Peptides, PbRII+, Pf1 and Pf2 all containing substantially Region II+ inhibited the binding of CS27IVC with the inhibition being complete at 250 μg/ml of peptide PbRII+.

Recombinant polypeptide Falciparum-1 which contains Region I and the repeats but not Region II+ did not bind to liver sections even at concentrations as high as 250 μg/ml.

Peptides Pf3, Pf4, Pf1A, Pf1B, Pf2A, Pf25C and Pf70 all failed to inhibit the binding of CS27IVC to liver sections even at concentrations as high as 500 μg/ml (Pf3).

These results indicate that both certain amino acids and their sequence within Region II+ are essential for binding to liver sections. The presence of two cysteines, for example, is important. Omission of N-terminal CSVT abrogates a substantial portion of the peptides' inhibiting ability. In fact, as will be shown below, the sequence CSVTCG (and its variants in Table 1) appear to be important for inhibition.

In order to quantify more accurately the effectiveness of various peptides in inhibiting the CS27IVC binding to hepatocytes additional experiments were performed using the human hepatoma cell line HepG2. This cell line is invaded readily by P.berghei sporozoites. Indirect immunofluorescence revealed that HepG2 cells bound recombinant CSFZ (Cys) but not falciparum-1. Incubation of HepG2 cells immobilized on the bottom of microtiter plates with increasing concentrations of CSFZ (Cys) showed that CSFZ (Cys) binds to these cells in a dose-dependent and saturable manner. The results, expressed in fluorescence units v. protein concentration are depicted in Figure 6. The open circles represent binding of CSFZ (Cys); the black circles represent binding of falciparum-1 (control). Saturation is projected in Fig. 6 at about 25 μg/ml CSFZ (Cys).

The same assay system was used to evaluate the ability of the various synthetic peptides to inhibit the binding of CSFZ (Cys) to the HepG2 cells. Paraformaldehyde-fixed immobilized HepG2 cells were pre-incubated with peptides at concentrations between 0 and 250 μg/ml, washed, and incubated with CSFZ (Cys) at 2.5 μg/ml. After washing, the cells were incubated with MAb 2A10 followed by anti-mouse IgG conjugated to alkaline phosphatase. Bound enzyme was revealed by a fluorescent substrate, methylumbelliferyl phosphate. Each peptide concentration was assayed in triplicate wells and the means of the fluorescent reading were calculated. The results were the same as in the frozen section experiments: Pf1 and Pf2 both inhibited the CSFZ (Cys) binding to HepG2 cells. Fifty percent inhibition was observed at peptide concentrations between 16 and 75 μg/ml, with

Pf1 (which includes both cysteines in Region II) showing the strongest inhibitive effect. The cysteines and the sulfhydryl groups were necessary for inhibition (Pf1A, Pf1B and Pf2A were not active). Pf70 which spans Region I and Pf25C had no inhibitory effect at concentrations as high as 250 μg/ml. The results are shown graphically in Fig. 7 wherein percent inhibition (after subtraction of background) is plotted as a function of peptide concentration. Pf1: open circles; Pf2: dark circles; Pf3: open upright triangles; Pf1A dark square; Pf1B open inverted triangles; Pf2A dark triangle; Pf70 open diamond; Pf25C dark diamond. Standard deviations were no greater than ±5% of the plotted mean.

Example 10: CS Binds to Microvilli of Hepatocytes in the Space of Disse

To identify the hepatocyte structures interacting with the CS, glutaraldehyde-fixed and LR white-embedded thin sections of rat and human liver were incubated with the recombinant CS polypeptide CS27IVC and analyzed by immunoelectron microscopy. Human liver tissue embedded in K4M was labelled with CS27IVC followed by MAb 2A10 and Protein A-gold.

Rat liver tissue was embedded in LR white and sections were labelled with CS27IVC, MAb 2A10 and goat-antimouse IgG - gold conjugate. Gold particles were found in areas of hepatocyte membrane exposed to the bloodstream, namely the microvilli protruding in the Space of Disse and to lateral membranes of adjacent hepatocyte up to the tight junctions that seal the bile canaliculi. Other regions of the hepatocyte plasma were not stained, and neither were cell membranes of Kupffer cells or endothelia. Liver sections incubated with falciparum-1 protein which lacks Region II+ or sections incubated only with monoclonal antibody 2A10 and/or with the gold conjugate were negative.

Intracellular hepatocyte labelling with CS27IVC was seen on the lysosomes in the vicinity of bile canaliculi possibly reflecting receptor internalization and degradation. The bile ducts themselves were negative. Lysosomal staining was also seen

in Kupffer cells and, occasionally, in cells containing lipid droplets. On the whole, labelling was extremely localized.

Rat liver sections incubated with Falciparum-2 instead, showed essentially the same results but with lower staining intensity. The results were the same in rat mouse and human livers. Other tissues showed no specific labelling pattern. The results were repeated even after homogenization and fractionation (by density gradient centrifugation) of hepatocytes. The membrane fractions showed staining, but other fractions were not stained.

The results of some of these staining experiments are shown in Figs. 3 and 4.

In Figure 3A, it is apparent that the CS protein binds the entire hepatocyte surface except for the area exposed to the bile canaliculi; in Figure 3B the binding of CS to the microvilli in the space of Disse is shown.

In Figure 4, it is apparent that the CS protein labels human lysosomes and also binds to the lateral hepatocyte cell membrane (arrowheads). (M stands for mitochondrium.)

In Figure 4B, lysosomes (L) of Kupffer cells (K) are labelled but not the Kupffer cell surface nor the endothelial cell membrane (arrowheads) whereas the hepatocyte microvilli in the space of Disse are heavily labelled.

In Figure 4C a rat liver cell membrane shows labelling of those membrane fragments that contain microvilli whereas other membranes (possibly bile canaliculi shown by arrowheads are not labelled and contaminating cell organ cells (*) are not labelled.

In Figure 4D, rat cell liver fractions containing mostly mitochondrium (M) and rough endoplasmic reticulum (arrowheads) are not labelled.

Example 11: Aggregated, Region II+-Containing CS Binds to Liver Membranes

The gold conjugates employed in the foregoing liver section experiments contained no aggregates. However, the staining pattern was always patchy which suggested that the receptors were clustered, or the CS was aggregated, or both.

Recombinant CS27IVC was subjected to molecular sieving as detailed above to isolate the monomeric form of the protein (which eluted between 66 and 200 kD) from the aggregated form (which eluted at 669 kD). The aggregated form of the protein retained binding activity whereas the monomure form was inactive. Aggregates of Falciparum-1 protein were tested as a control and were found inactive, as expected.

Falciparum-1 aggregates were formed by introduction of sulfhydryl groups followed by air oxidation. The presence of aggregates or monomers in the active and inactive fractions, respectively, was confirmed by SDS-PAGE under reducing and nonreducing conditions followed by Western blotting using MAb 2A10. Reducing conditions resulted in the observation of a single band in all samples. Nonreducing conditions preserved several bands of increasing molecular weight in the FPLC fraction that had showed activity. (See, Example 5 above). The results are depicted in Figure 5 which is a plot of OD v. FPLC fraction number. It shows that the CS protein eluted in two peaks, with the first one (fraction 9) corresponding to the aggregated form and the second one (Fractions 12-14) corresponding mainly to monomeric forms.

Example 12: Peptide PbRII+ and Anti-RII+ Antibodies Inhibit Sporozoite Invasion of HepG2 Cells

HepG2 cells were incubated with P.berghei sporozoites in the presence of varying amounts of P.berghei peptide PbRII+ or control peptides. The number of exoerythrocytic forms ("EEF") of the parasite that developed in the HepG2 cells were counted two days later. Because the viability and infectivity of sporozoites vary greatly, multiple experiments were performed so the results are statistically significant. HepG2 cells were plated at a density of 0.5 x 10^6/ml, incubated for two hours with 50,000 P.berghei sporozoites per well in the presence of PbRII+ or other control peptides, or simply media, as indicated in Table 2 below. Cultures were grown for two days, fixed and stained with MAb 2E6 followed by goat anti-mouse IgG conjugated to horseradish peroxidase. In Table 2, "Number of EEF" represents the average

number of schizonts counted per 20 fields under 20-times magnification of a light microscope in quadruplicate wells; P values were calculated by one-way analysis of variances corrected by the Bonferroni method using the commercially available computer program GraphPAD Instat, version 1.14, copyright 1990. In Experiment 6, peptides were added to the culture 2 hours after the addition of sporozoites, at which time invasion was complete.

TABLE 2

PbRII+ Inhibits P.berghei Sporozoite Invasion

Experiment	Inhibitor Peptide	Concentration (μg/ml)	Number of EEF[a] (Mean ± SD)	Percentage of Inhibition	P Value[b]
1	PbRII+	250	53.7 ± 3.1	84.2	<0.01
	PbRII+	125	242.7 ± 23.8	28.5	NS
	PCD59 (control)	250	250.3 ± 103.2	26.3	NS
	Medium alone	---	339.7 ± 52.4	-	
2	PbRII+	250	29.3 ± 8.6	66.8	<0.01
	Pf4 (control)	250	122.5 ± 13.4	0.0	NS
	Medium alone	---	88.3 ± 9.3	-	
3	PbRII+	250	35.7 ± 11.2	74.9	<0.01
	Pf4 (control)	250	123.7 ± 20.1	13.2	NS
	Medium alone	---	142.5 ± 34.8	-	
4	PbRII+	250	27.2 ± 4.8	81.5	<0.001
	Pf4 (control)	250	105.0 ± 12.5	28.1	NS
	Medium alone	---	146.0 ± 28	-	
5	PbRII+	500	0.0	100.0	<0.001
	Pf4 (control)	500	375.2 ± 52	0.0	NS
	Medium alone	---	370.0 ± 47	-	
6[c]	PbRII+	250	541.0 ± 186	0	NS
	Medium alone	---	523.5 ± 84	0	

HepG2 cells were plated at a density of 0.5 x 10^6/ml, incubated for 2 hr. with P.berghei sporozoites (50,000 per well) in the presence of the PbRII+, negative control peptides (Pf4 and CD59), or media alone. Cultures were grown for 2 days, fixed, and stained with MAb 2E6, followed by goat anti-mouse immunoglobulin conjugated to horseradish peroxidase. Numbers in the fourth column represent the average schizonts counted per 20 fields under 20 x magnification of a light microscope in quadruplicate wells (± the standard deviation).

[a] EEF = exoerythrocytic forms.

[b] P values were calculated using one-way analysis of variance (ANOVA), corrected by the Bonferroni method. NS, not significant.

[c] In this experiment, peptides were added to the culture 2 hr. after the addition of the parasites, after completion of invasion.

As can be seen in Table 2 PbRII+ was effective in inhibiting EEF if it had been present during the initial phases of invasion. Two hours after addition of the sporozoites PbRII+ was no longer effective.

Several rabbits were hyperimmunized with PbRII+ conjugated to keyhole limpet hemocyanin. The antiserum titer of the animals was high (≥20,000 by ELISA). Nevertheless, only one antiserum reacted weakly with sporozoites (1:1000 by indirect immunofluorescence). The IgG fraction of this antiserum at 700 μg/ml significantly inhibited sporozoite invasion of HepG2 cells while preimmune IgG had no effect. Again the IgG fraction of immune sera was ineffective 2 hours after exposure of the cells to sporozoites. Thus, the ability of PbRII+ to inhibit binding of CS to the hepatocyte receptor was confirmed. The results of the IgG experiments are set forth in Table 3 below. Again, the 6th experiment involved delayed addition of the anti-PbRII+ antisera.

TABLE 3

Anti-PbRII+ Inhibits P.berghei Sporozoite Invasion

Experiment	Antibody	Concentration (mg/ml)	Number of EEF[a] (Mean ± SD)	Percentage of Inhibition	P Value[b]
1	Anti-RII+	2.8	45.7 ± 22.6	84.4	<0.01
	Preimmune	2.8	295.0 ± 68.8	0.0	NS
	Medium alone	---	293.5 ± 34.5	-	
2	Anti-RII+	2.8	93.0 ± 76	70.6	<0.01
	Anti-RII+	1.4	111.3 ± 8.1	65.8	<0.05
	Anti-RII+	0.7	166.2 ± 38.4	47.4	<0.05
	Preimmune	2.8	243.7 ± 38.9	23.1	NS
	Medium alone	---	316.0 ± 76.9	-	
3	Anti-RII+	2.8	299.5 ± 26.1	46.1	<0.01
	Medium alone	---	555.5 ± 7.7	-	
4	Anti-RII+	2.8	187.3 ± 71	57.4	<0.01
	Preimmune	2.8	347.5 ± 13.4	21.0	NS
	Medium alone	---	440.0 ± 59.9	-	
5[c]	Anti-RII+	2.8	289.5 ± 25	16.5	NS
	Preimmune	2.8	344.0 ± 43.8	0.6	NS
	Medium alone	---	346.2 ± 13	-	
6[c]	Anti-RII+	2.8	283.0 ± 45.3	27.2	NS
	Preimmune	2.8	316.2 ± 88.5	18.6	NS
	Medium alone	---	388.7 ± 41.4	-	

HepG2 cells were plated at 0.5 x 10^6/ml, incubated for 2 hr with P.berghei sporozoites (50,000 per well) in the presence of protein A-purified anti-PbRII+ IgG, preimmune IgG, or medium alone. Cultures were grown for 2 days, fixed, and stained with MAb 2E6, followed by goat anti-mouse immunoglobulin conjugated to horseradish peroxidase. Numbers in the fourth column represent the average number of schizonts counted per 20 fields under 20 x magnification of a light microscope in quadruplicate wells (± the standard deviation).

[a] EEF = exoerythrocytic forms.

[b] P values were calculated using one-way analysis of variance (ANOVA), corrected by the Bonferroni method. NS, not significant.

[c] In this experiment, peptides were added to the culture 2 hr. after the addition of the parasites, after completion of invasion.

Examples and Utility of Peptides According to the Invention

The foregoing experiments show that CS, in fact aggregated CS, recognizes specifically the basolateral domain of hepatocyte cell membrane. This specificity predicts the existence of a receptor on the hepatocyte cell surface. The ligand for this receptor (which is characterized below) resides within Region II+ of the CS protein, as defined by the present inventors.

Although the foregoing experiment focused on P. falciparum Region II+, the units are expected to be the same with Region II+ prepared from other species.

In fact, the peptide Pf1, an example of a peptide consisting essentially of Region II+ competes very effectively with recombinant CS and (at micromolar concentrations) with the P.berghei sporozoites. The peptide Pf2 which lacks the amino acids PCSVT competes much less effectively indicating that the missing sequence is part of the adhesion ligand (nevertheless, peptide Pf2 also binds to the same site as the CS protein). The cysteines in Region II+ are also important because analogs of Pf1 lacking only these cysteines were totally inactive.

Additional suitable peptides containing essential parts, or the entirety, of Region II+ can be easily identified using one or more of the above-described assays and the overlapping peptide method, which is a peptide screening technique well-known in the art and no more than routine experimentation. With reference to Table 1, peptides formed by omitting progressively one-by-one C-terminal amino acids from Regions II+ of different malarial species can be tested for CS-binding inhibitory activity. (It has already been determined that the N-terminal of Region II+ is important. The only remaining questions fall into the category of whether, e.g., E W T P C S V T C G V G V derived from P. vivax Region II+ would have inhibitory activity and whether the higher order structures (e.g., the small peptide loop formed by sulfide bonds beteen the two cysteines) are essential for inhibitory activity.

Depending on the use for which they are intended. peptides and peptide-containing constructs within the present invention include the following:

(A) Peptides that inihibit the binding of CS protein (in this context "CS protein" includes recombinant CS polypeptides and entire sporozoites) by simply competing for receptor sites on hepatocytes. Such peptides should possess substantial CS-binding inihibitory activity (e.g., not substantially less than that of Pf2) and, if intended for use in vivo, should not elicit a substantial immune response from the host against the immunodominant (repetitive) epitope of the CS protein.

Such peptides may be as small as the minimum CS-binding inhibitory amino acid sequence from Region II+ or as large as CSFZ, i.e., consisting essentially of the CS-protein minus the immunodominant region. Such peptides should be soluble in aqueous media. Region II of Dame is excluded from the present invention.

The formation of disulfide-bond linked oligomers or aggregates of Pf1 (or CS) using the two cysteine residues probably imparts optimal binding activity, and the present inventors have evidence that Pf1 forms aggregates. Accordingly, peptides within the invention that compete for binding with CS should preferably be aggregated. This is consistent with the finding that CS aggregates bind to hepatocytes and CS monomers do not.

Even though Region II+ is not itself particularly immunogenic, non-immunogenic peptides incorporating it are nevertheless useful in inhibiting sporozoite invasion of hepatocytes. In fact, the absence of immunogenicity of Region II+ can be used to advantage in vivo, because the host to whom such peptides are being administered will not mount an immune response against them.

For example, Region II+-containing peptides can be administered to malaria susceptible subjects, for example intravenously, at sufficiently high concentrations to compete effectively with a subsequent challenge with sporozoites. These

concentrations can be determined by means known to one of ordinary skill in the art. For example, optimum concentrations can be established using serially diluted preparations of the peptide in connection with a suitable testing procedure. Preferred concentrations range from about 1 to about 10mg in a mouse and from about 10 to about 100 mg in a human. Suitable vehicles for administration include, but are not limited to, isotonic saline. The peptides of the present invention can also be encapulated in liposomes, the encapsulation having been described by Brenner, D., J.M.C.I. 78:1436, 1989; Anderson, P. et al., Cancer Research 50:1853, 1990; and Anderson, P. et al., J. Immunotherapy 12:19, 1992.

Peptides consisting of Region II+ or of the ligand adhesion portion thereof, are not expected to be toxic to the malaria-susceptible mammalian hosts because they were not toxic when administered to mice.

Such administration of Region II+ peptides would have to be preventive because sporozoites are targeted to the liver of the infected mammal so efficiently and quickly that therapeutic administration would not be effective, as shown by Example 12 above. Simultaneous infection and administration is about the limit of the CS-binding inhibitory effectiveness of Region II+ peptides.

(B) Alternatively, Region II+ peptides could be used to raise antibodies in vitro. These can be human or humanized monoclonal antibodies. Human origin or humanization would cause the immune system of the host to be "blind" to the antibodies, and raising them against Region II+ would cause them to bind to the ligand adhesion site of the CS protein (or sporozoites) and thus prevent liver invasion through the hepatocyte receptor. Human chimeric and humanized antibodies of various predetermined specificities are engineered currently, See, e.g. Presta, L.G. Curr. Op. Struct. Biol. 2:593-596, 1992; and Burton, D.R., Hospital Practice, August 15, 1992, 67-74 and references cited in each. See also Barbas, et al., Proc. Nat'l. Acad. Sci. USA 88:7978, 1991. The amount of monoclonal antibody administered should be sufficient to achieve a blood level ranging from about

1 to about 10 mg/ml. In vitro elicitation of antibodies can be performed according to methods known to those skilled in the art.

(C) The nonantigenic nature of Region II+ does not preclude use of peptides containing Region II+ (and also containing an antigenic determinant) in vaccines.

For example, peptides consisting essentially of Region II+ and the immunogenic amino acid sequences immediately following or preceding Region II+ of the CS protein can be used to immunize susceptible hosts. (See, Good et al., Annu. Rev. Immunol. 6:663-688, 1988).

In P.falciparum, such an antigenic C-proximal amino acid sequence is the sequence N K P K D Q L D Y Q N D I Q.

Other examples of such antigen sequences include, but are not limited to:

1. P S D K H I E Q Y L K K I K N S I (TH2R),
2. P S D Q H I E K Y L K R I Q N S L (TH2R), and
3. D K S K D Q L N Y A (TH3R).

See, e.g., Nussenzweig, V. and Nussenzweig, R., September 15, 1990, Hospital Practice 45-57; Good, M.F., et al., Annu. Rev. Immunol. 6:563, 1988). Although polymorphic, TH2R and TH3R include only a few interspecies amino acid substitutions, and therefore, polymorphism would not be an impediment to incorporating them in a malaria vaccine preparation.

In addition, Region II+ peptides would be useful in drug design, i.e. in the construction of molecules (for use in chemoprophylaxis) that bind to the CS hepatocyte receptor with sufficient affinity to inhibit the subsequent binding of sporozoites. An in vitro assay system for this purpose has been largely described above. It could employ for example HepG2 cells as targets and would test the ability of recombinant CS proteins to bind to their receptors in the presence or absence of a designed putative drug based on Region II+. The drugs which inhibit this binding would then be tested for their effectiveness in inhibiting sporozoite invasion of HepG2 cells.

Peptides consisting essentially of Region II+, or its ligand adhesion subregions, can also be used for such drug screening and are more covenient for this purpose than recombi-

nant CS-constructs. Such Region II+ derived peptides could be labelled and used exactly as the recombinant CS protein, or their binding to hepatocytes could be assessed previously, and the unbound peptide could be measured.

The Hepatocyte Membrane Receptor for CS Protein

The existence of a receptor for the CS protein in the basolateral domain of hepatocyte plasma membranes has been shown in the experiments by the localization and specificity of the binding and the resemblance of the foregoing experimental results to other receptor-ligand interactions involving different proteins and their receptors.

The nature of this receptor is not known, but there are indications that it may be a sulfated macromolecule and most likely a heparan sulfate proteoglycan. For example, other proteins bearing the Region II+ motif bind specifically to sulfated compounds. Recombinant CS proteins from P. vivax (vivax-1) and P. falciparum (falciparum-1) containing Region II+ (but not proteins lacking Region II+ such as vivax-2 and falciparum-2) also specifically bind to sulfatides as well as to cholesterol-3-sulfate. This binding is abrogated by reduction or alkylation of the recombinant CS constructs. This suggests that the two cysteines in Region II+ form a disulfide bond forming a small peptide loop which appears important in receptor recognition.

In addition, the binding of the recombinant constructs as well as live sporozoites is inhibited by dextran sulfate, which makes it likely that the receptor is a sulfated glycoconjugate. To exert its full inhibitory function, the dextran sulfate should be present before contact of the CS polypeptide (or of the sporozoites) with the hepatocytes (in much the same manner as described in Example 12 above for the inhibitory ability of Region II+ peptides present in the extrahepatocytic medium prior to challenge with live sporozoites).

Finally, CS polypeptides do not bind to gangliosides or steroids that are structurally similar to sulfatides and cholesterol sulfate but are not sulfated; and they do not bind to polyanionic compounds (including sulfated polyanionic compounds)

such as heparan-sulfate, chondroitin-sulfate, and hyaluronic acid.

Lastly, dextran does not appreciably inhibit sporozoite invasion of hepatocytes, which is consistent with the conclusion that a sulfated receptor structure is involved.

Although the structure of the CS receptor is of importance in drug design, one of the advantages of the present invention is that it does not regain knowledge of the receptor structure.

The peptides or constructs should have no substantial ability to elicit the formation of antibodies recognizing the immunodominant epitope of CS that would diminish the effectiveness of the peptides or constructs.

WHAT IS CLAIMED IS:

1. A peptide inhibitor for the binding of a circumsporozoite polypeptide to receptors of hepatocytes from malaria-susceptible mammals, said inhibitor having an amino acid sequence selected from the group consisting of:

(i) Region II+ of a circumsporozoite protein, said Region II+ containing the subsequence CSVTCG;

(ii) fragments of said Region II+ containing at least a portion of the adhesion ligand for said receptors, said portion comprising at least one cysteine of said Region II+;

(iii) peptide constructs comprising (a) (i) or (ii) and (b) at least one other fragment of the amino acid sequence of said circumsporozoite protein, said constructs having no substantial ability to elicit the formation of antibodies recognizing the immunodominant epitope of said circumsporozoite protein.

2. A peptide inhibitor for the binding of a circumsporozoite polypeptide to basolateral plasma membrane of hepatocytes from malaria-susceptible mammals, said inhibitor having an amino acid sequence selected from the group consisting of:

(i) Region II+ of a circumsporozoite protein, said Region II+ containing the subsequence CSVTCG;

(ii) fragments of said Region II+ containing at least a portion of the adhesion ligand for said receptors, said portion comprising at least one cysteine of said Region II;

(iii) peptide constructs comprising (a) (i) or (ii) and (b) at least one other fragment of the amino acid sequence of said circumsporozoite protein, said constructs having no substantial ability to elicit the formation of antibodies recognizing the immunodominant epitope of said circumsporozoite protein.

3. A method of inhibiting the binding of a circumsporozoite polypeptide to hepatocytes susceptible to sporozoite invasion comprising:

supplying to the environment of said hepatocytes an amount effective to inhibit said binding of the peptide inhibitor of claim 1, no later than exposure of said hepatocytes to said circumsporozoite protein.

4. The method of claim 3 wherein said circumsporozoite polypeptide is native circumsporozoite protein present on the surface of viable, infectious malarial sporozoites.

5. The method of claim 3 wherein said circumsporozoite polypeptide is selected from the group consisting of native circumsporozoite protein, recombinant circumsporozoite protein, and recombinant fragments of circumsporozoite protein each comprising Region II+.

6. The method of claim 3 wherein said hepatocytes are human hepatocytes.

7. A peptide consisting essentially of Region II+ of the circumsporozoite protein.

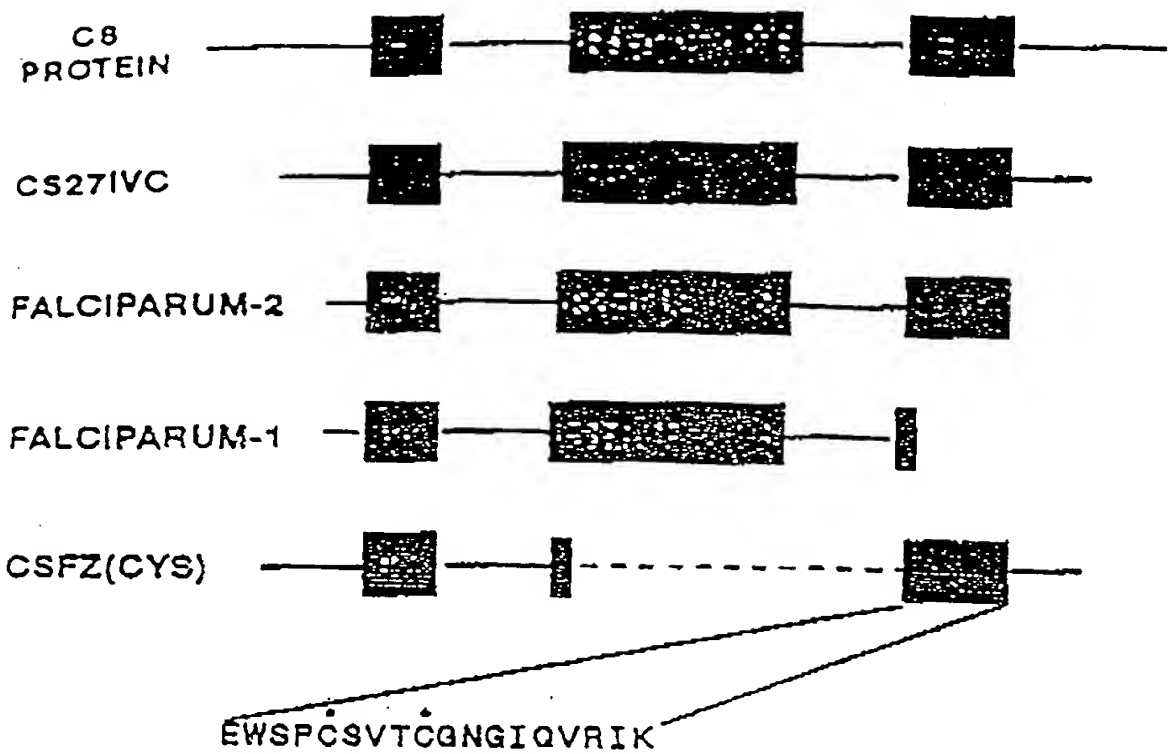
8. The peptide of claim 7, said Region II+ being selected from the group consisting of

E W S P C S V T C G N G I Q V R I K P G S A;
E W T P C S V T C G V G V - - R V R;
E W S P C S V T C G S G I R A R R K;
E W S P C S V T C G K G V R M R R K;
E W S P C S V T C G K G V R M R R K;
E W S P C S V T C G S G I R A R R K;
E W S Q C N V T C G S G I R V R K R;
E W S Q C S V T C G S G V R V R K R; and
E W S E C S T T C D E G R K I R R R;

9. The peptide of claim 8, said peptide being
P C S V T C G N G I Q V R I K P G S A.

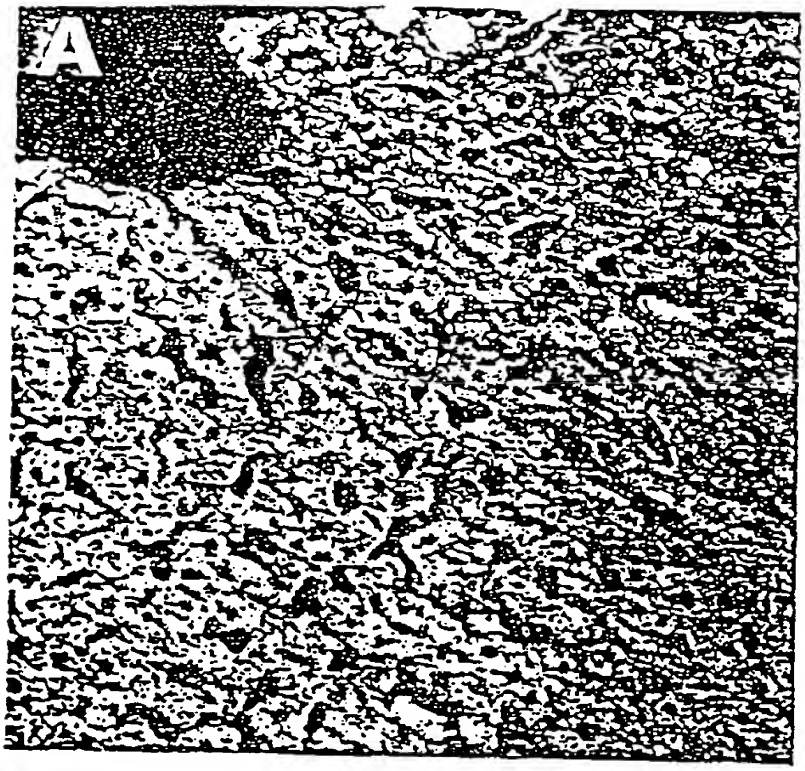
10. The peptide of claim 7, said peptide being P C S V T C G N G I Q V R I K.

11. The peptide of claim 8, said peptide being P C S V T C G V G V R V R.



pepPf1	PCSVTCGNGIQVRIKPGSAN
pepPf1δ	PXSVTXGNGIQVRIKPGSAN
pepPf2	CGNGIQVRIKPGSANKPKDE
pepPf2A	AGNGIQVRIKPGSANKPKDE
pepPf3	PGSANKPKDELDYANDIEKK
pepPbRII	CNVTCGSGIRVRKRKGSNKKAEDL
pepPf70	KPKHKKLKQPADGNPDPNAN

FIG 1



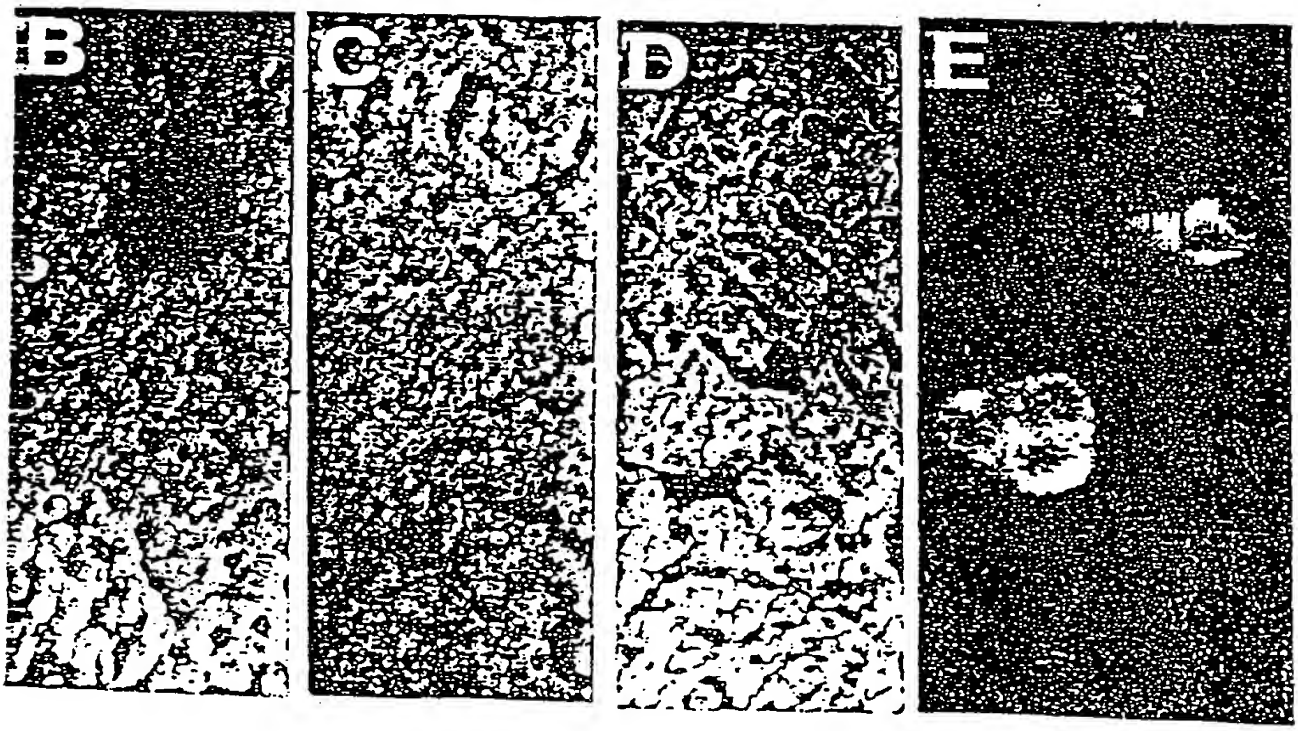


FIG. 2

FIG. 3

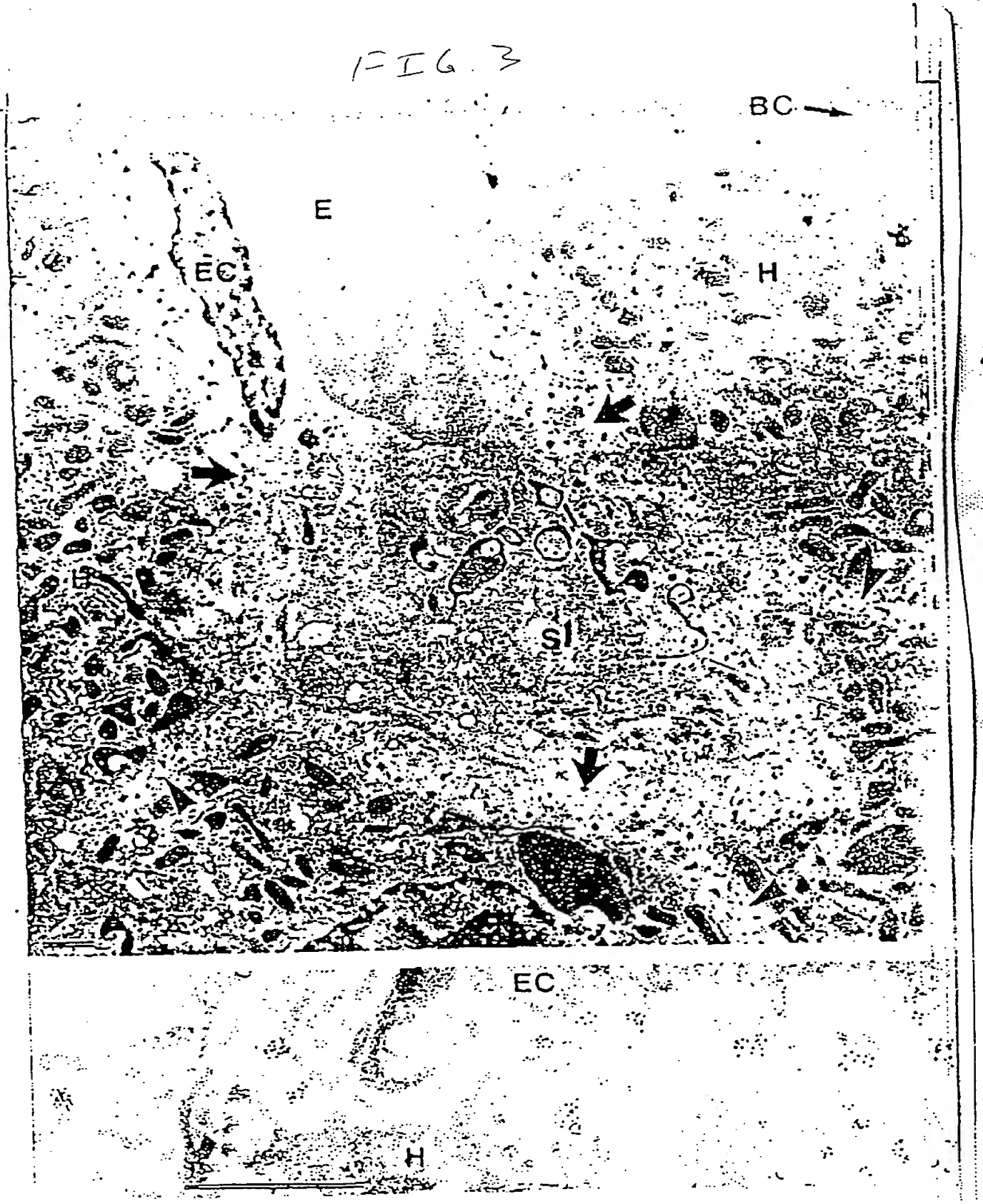
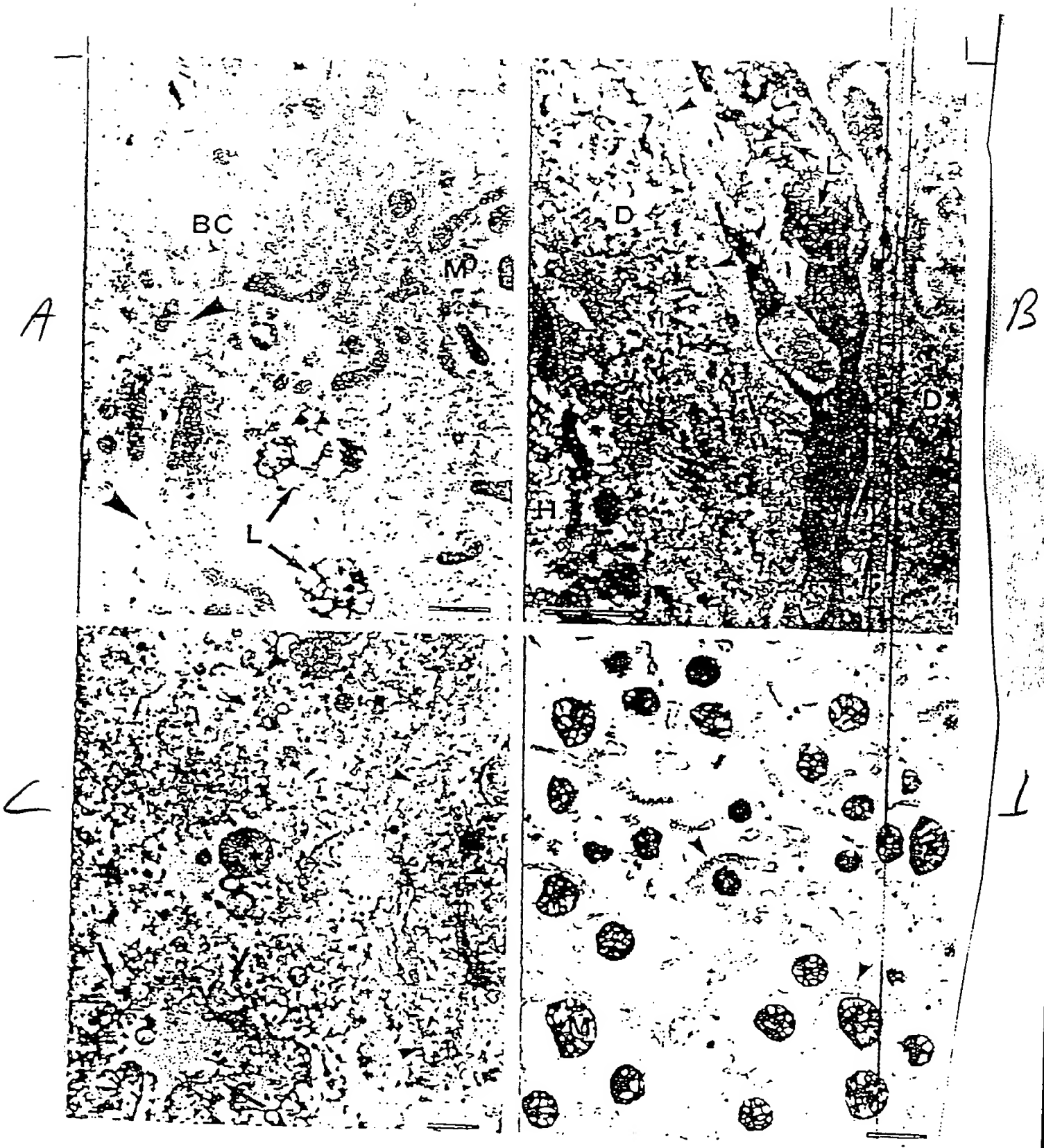


FIG 4



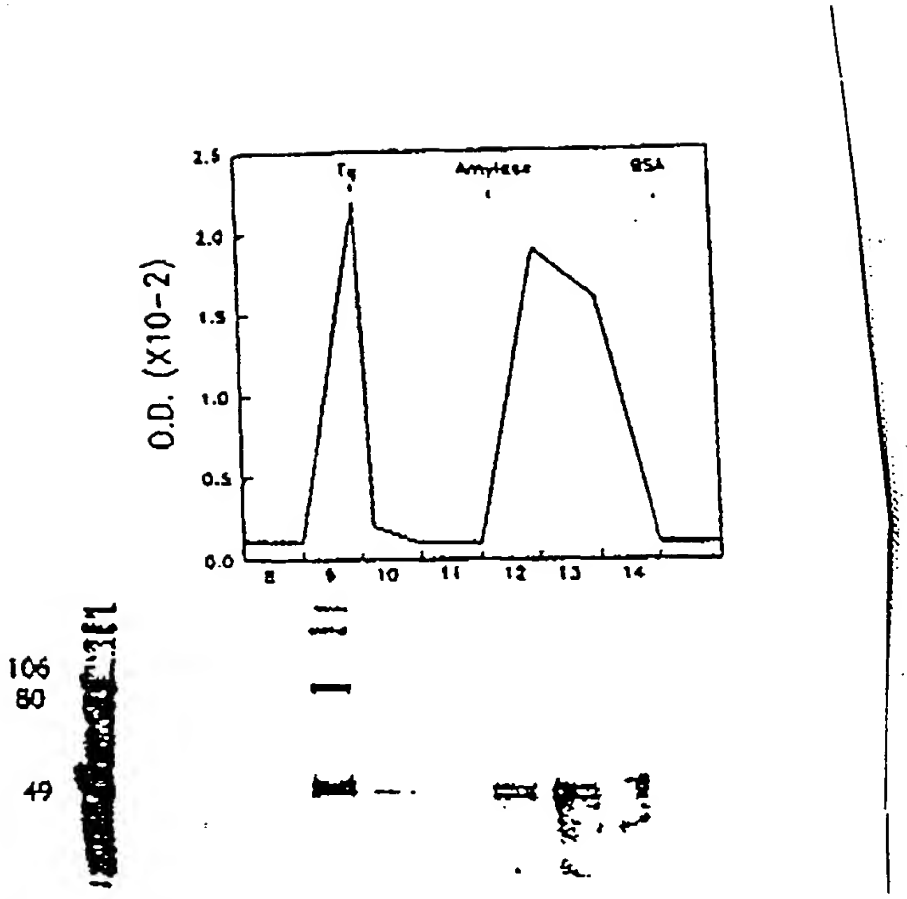


FIG. 5

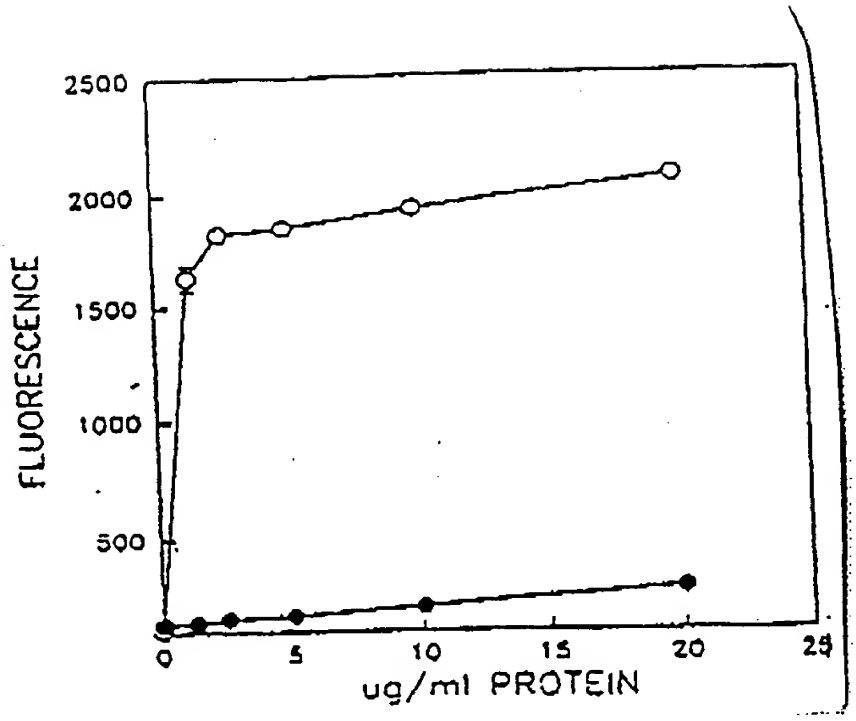

2500
2000
1500
1000
500
FLUORESCENCE
0
5
10
15
20
25
ug/ml PROTEIN

FIG. 6

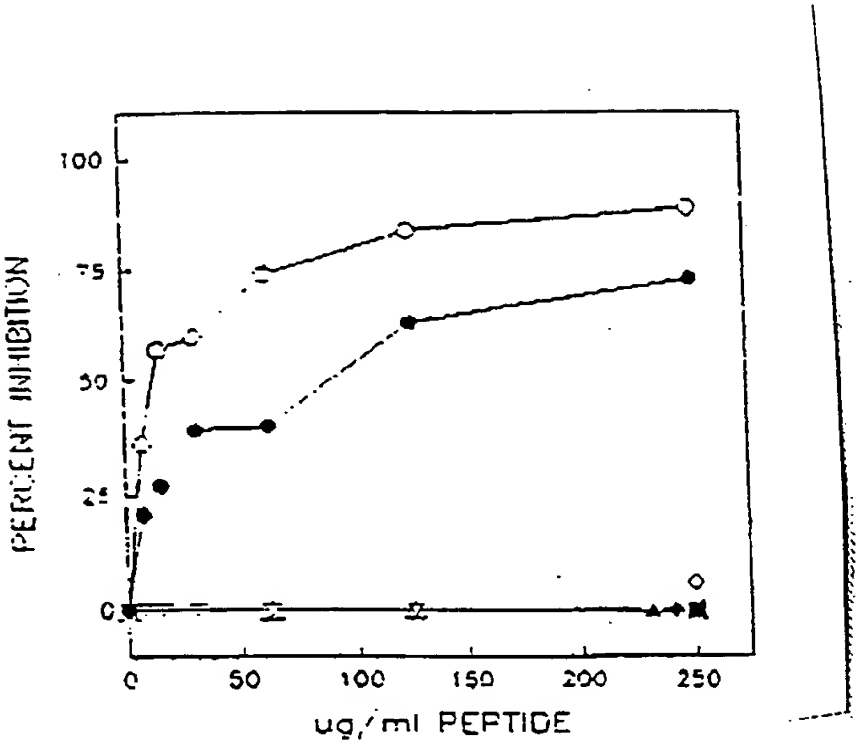

PERCENT INHIBITION
100
75
50
25
0
0
50
100
150
200
250
ug/ml PEPTIDE

FIG 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08800

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/002; C07K 7/08, 7/10, 13/00
US CL :514/2, 8, 12; 424/88; 530/300, 324
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 8, 12; 424/88; 530/300, 324

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated Patent System; DIALOG files 155, 399, 5, 351.
Search terms: inventor's names; Plasmodium(w)falciparum, sporozoite, hepatocyte

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Synthetic Peptides in Biology and Medicine, issued 1985, E. Ruoslahti, et al., "Synthetic peptides in analysis of cell adhesion", See pages 191-197 and abstract.	1-11
Y	J. Exp. Med., Vol. 164, issued December 1986, Aley, et al., "Synthetic peptides from circumsporozoite proteins of Plasmodium Falciparum and Plasmodium Knowlesi recognize the human hepatoma cell line HepG2-A16 in vitro", See pages 1915-1922.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 1993	NOV 12 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer Thomas M. Cunningham Telephone No. (703) 308-0196

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